An Evaluation of Polymeric Nanoparticles as an Alternative for Vaccinations

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Introduction

Although many studies have been done on antibodies and vaccines, little is known about the transfection methods of nanoparticles capable of carrying antigens into cells to create effective mRNA vaccines (Garmory, 2003). This intricate process of introducing foreign mRNA into a body to provide immunity is grounded in two overall processes, translation and transfection. Both of these processes are studied in depth in immunology research, which is essentially the study of the immune system. Translation allows for gene expression and antigen production. Research has introduced a relatively new concept within the last 20 years known as transfection. Transfection is the deliberate introduction of naked nucleic acids to eukaryotic cells in hopes of them translating and replicating properly within the body. Since the introduction of foreign objects is often rejected by the body, transfection is often prevented by, in theory, cellular stress.

Thus far in immunotherapy, vaccines allow for short term production of the desired proteins in vivo (Delany, 2014). Currently, long term or even life-long immunity cannot be provided by vaccines with few exceptions such as the measles vaccine. Even with the idea that the effects of current vaccines are short term, side effects can still occur and inconvenience patients, or even cause some mild to severe form of illness.

This is particularly relevant now with the introduction of COVID-19 vaccines that feature lipid nanoparticles with mRNA. (Polack et al, 2020) Although mRNA vaccines have been recognized for their rapid development potential, high potency, and lower production costs (Pardi et al, 2018), there are still many considerations that must be made. Is a lipid nanoparticle when compared to polymeric nanoparticles the best choice for mRNA vaccines? To address this question, two key factors, vaccine tolerability and persistence will be explored.

To define persistence, an effective mRNA vaccine will give people innate immunity so that the inconvenience of receiving a vaccine every year would be diminished, if not completely removed. Potentially, the vaccine could also be effective enough to remove the need for booster shots. Currently, the lipid nanoparticle based COVID-19 vaccines require a booster shot, and it is currently unknown as to how long the provided immunity lasts according to the CDC. (Centers for Disease Control and Prevention, 2021) Tolerability would be defined as the degree of incidence of side effects. Over 80% of participants in the BNT162b2 Pfizer clinical trials experienced some degree of side effects and around 16% of participants younger than 55 reported fevers. (Polack et al, 2020) According to the World Health Organization (WHO), vaccines such as inactivated influenza vaccines only have a <5% chance to induce fevers in adults and only 64% of adults experience mild side effects such as soreness. As such, it is important to the difference in tolerability between these different types of vaccinations. In this paper, we will primarily be focusing on evaluating polymeric nanoparticles as an alternative for vaccinations.

To observe the potential effectiveness of these polymer nanoparticles, several experiments were run. Since translation is such an intricate and quick process with many steps, it is difficult to determine the location in the process that transfection can fail when mRNA is placed in the body via injection. Introducing foreign mRNA to a cell often causes cellular stress, which can be observed using numerous tests. Various issues could potentially be the reason for this such as reactive oxygen species, reactive nitrogen species, or even stress granules among other things (Buchan 2009). All of these are produced by cells when they are stressed by external factors such as the polymeric nanoparticles we will be using to test these individual steps on the pathway. The true and specific cause of transfection shutdown remains to be seen.

Every year, at least 200,000 are hospitalized due to the flu in the U.S. alone. If proper transfection with nontoxic nanoparticles loaded with the desired mRNA can be achieved, an effective mRNA vaccine can be created. If done properly, polymer nanoparticles with mRNA being used in a vaccine could reduce side effects commonly associated with the lipid nanoparticle mRNA vaccines that are currently available and potentially save millions of lives. The purpose of our experiments is to find where exactly transfection fails in polymeric nanoparticles with mRNA to bring us one step closer to our goal. Each of our experiments target and observe a particular step to ensure that the DNA and RNA are working properly before shutdown mechanisms intervene.

Literature Review

Vaccines are some of the most effective means of preventing certain infections and have even wiped out some diseases such as smallpox. Traditionally, they work by essentially placing weakened microorganisms that can still prompt immune responses without causing disease into the body. An ideal vaccine would be safe for all patients, long lasting, cheap, and provide lifelong immunization to those that receive them. However, they also have limitations that prevent them from being as effective or safe for people as they could be. These limitations include having long, complicated production cycles, limited production capacities, and the potential to prompt allergic responses or side effects (Du, 2010). Even vaccines that feature lipid nanoparticles such as the COVID-19 vaccines have many of the same limitations and require storage at ultra-low temperatures to maintain their effectiveness. (Schoenmaker, 2021)

As such, polymeric nanoparticles have been a subject of interest in vaccine and drug research for some time. Some advantages that they have over lipid nanoparticles include

enhanced stability, unique customizability, and a more sustained release system. They also do not require the same level of extreme environmental care to ensure their effectiveness. Because of these characteristics, polymeric nanoparticles can increase circulation time of a drug throughout the body. (Guo et al, 2019)

Of the numerous polymeric nanoparticles, chitosan has been studied the most out of the natural subset. It is known in particular for its biocompatibility, biodegradability, non-toxicity, and ability to be easily modified. This modifiability is due primarily to its positive charge. (Rezigue, 2020) For our experiments, we used imidazole modified chitosan, also known as chitosan-IAA. This modification has been shown to demonstrate enhanced endosomal escape via enhanced solubility and buffering capacity. (Ghosn, 2009) However, chitosan has also been known to provide low immunogenicity despite all of the other listed advantages. This could be due in part to chitosan's low mRNA expression, in other words, lack of transfection.

One of the most current hypotheses is that the mRNA cannot transfect due to cellular stress and shutdown mechanisms. Many substances are produced within or by a cell when cellular stress is induced, and any one of these could potentially be the indicator or reason why the process of transfection cannot occur. Examples of substances that have been and will be studied include ROS (Reactive Oxygen Species), RNS (Reactive Nitrogen Species), and stress granules among others. Reactive oxygen species (ROS) encompass a variety of molecules; some examples include the hydrogen atom, with one unpaired electron, most transition metal ions, nitric oxide, and oxygen with two unpaired electrons. (Fruehauf, 2007) Reactive nitrogen species (RNS) are radicals like nitric oxide and nitric dioxide, as well as non-radicals like nitrous acid and dinitrogen tetroxide. (del Rio, 2015) Stress granules are essentially groups of untranslating mRNA that stalled in the process of translation due to cell stress. (Protter, 2016) If any one of

these particular substances is found to be the reason that transfection cannot occur, the next step would be to find out how to shut down that process for a short time to allow the mRNA to properly replicate and provide the desired lifelong immunity with minimal, if any, side effects.

Overall, the first step is the find the reason why mRNA is not transfecting. The next would be to find out how to shut down said step to allow for transfection. If these two are accomplished, the idea of an effective mRNA polymeric nanoparticle vaccine would be possible and provide people greater immunity with fewer side effects.

Methodology:

Each of the following protocols pertain to some aspect of transfection that we are observing and gathering data from. Each protocol looks at one step on the transfection pathway and we are trying to see which step causes transfection to fail.

Uptake experiment Protocol:

This particular protocol is used after each step to see how many of the cells have uptaken the particular component that we are observing.

On day one, split the cells first. For clarification, splitting the cells means to effectively throw away half of the cells and keep the other half. This prevents cancer cells like HELA from overgrowing and dying due to the rapid increase in cells fighting for a limited amount of nutrients. For cell splitting (HELA), which we need to do about twice a week, we use the following steps. First, observe cells under microscope to make sure they are ready to split and at around 90% confluence. Thaw an aliquot of trypsin from the fridge. Trypsin is an enzyme that releases cells from the surface of the flask. Aspirate off media. Wash cells by adding 10 mL 1x sterile PBS and aspirating. For a T75 flask, add 2 mL of trypsin (enough to cover the surface of

the flask). Move flask to the incubator and wait 5 minutes. Observe cells under microscope. The cells should be floating. If they aren't, move back to the incubator and wait 5 minutes. Add 8 mL of complete medium to neutralize trypsin. Centrifuge cells at 500 g for 5 minutes. Aspirate out the media and count the cells using AOPI dye. Put about ~250000 cells in two flasks and however much media needed to reach 15 mL in the flask.

For day 2, begin by creating particles at desired concentrations. Wash the media out with PBS 3 times. Add the fluorescent mRNA (Cy5) to the particles. Put the particles at the chosen concentrations into the wells. Allow to incubate for chosen timepoint (3 hours). Read uptake with flow cytometer using the flow cytometry uptake procedure. At uptake timepoints, wash plate with PBS 3x. Add 50 uL trypsin and incubate at 37 C for 5 minutes. Remove cells to FACS tubes. Add 1 mL FACS buffer to each tube. Centrifuge at 500 g for 5 minutes. Fix the cells using the cytofix. Discard supernatant and add 250 uL Cytofix (4% PFA). Add 1 mL FACS buffer to each tube. Centrifuge at 500 g for 5 minutes. Resuspend in 200 uL FACS buffer and read on Accuri(APC channel).

Stress Granule Experiment Protocol:

This experiment looks at the relationship between stress granules and transfection.

On day one, seed cells in 96 well plate (or whatever size is needed) at concentration of 10,000 cells/well. Have at least 4 wells per sample concentration and allow 24 hours for cells to adhere to plate.

To make chitosan-IAA, start by adding .2 mL of TPP to 480 microL of acetate buffer. Mix 250 microL of chitosan-IAA (at 4 mg/mL) with 250 microL of acetate buffer. Add the

mixtures together. Rotate the mixture for 30 minutes at room temperature. Centrifuge at 4000 g for 20 minutes. Resuspend in 1 mL of PBS.

To make bPEI-IAA, take .1 mL of bPEI-IAA stock solution (3 mg/mL) and make a TPP solution. Add 200 micrograms of TPP (20 microliters) to 880 microliters of DI water. Vortex then place on rotator for 30 minutes. Then centrifuge as detailed in the Chitosan-IAA protocol and resuspend.

Reactive Oxygen Species:

This experiment looks at the relationship between ROS and transfection.

For the ROS experiments, the procedure was done as follows: The day before the experiment, a cell well plate is seeded with about 30,000 HELA cells/well. Leave them overnight to attach properly. The next day, wash cells with 175 microliters of FACS buffer and flick the plate quickly and powerfully to remove the supernatant effectively. Stain the cells with 100 microliters 25 microM DCFDA in 1x FACS buffer for 45 minutes at 37 degrees Celsius. In order to make the solution, add 15 microliters of DCF-DA stock to 6 mL of FACS buffer. Next, prepare 5 mM of H2O2 to use as the positive control. Then wash the cells once with the same amount of FACS buffer as before. Flick the plate again to remove the supernatant. Add 150 microliters of FACS buffer to each well. Then read the 0hr fluorescence using the Gain60 protocol on the Gen5 microplate reader. Treat the cells. Then read the fluorescence at all the desired timepoints. To normalize, divide fluorescence from each time point by the 0hr timepoint data.

Reactive Nitrogen Species:

This experiment looks at the relationship between RNS and transfection.

The procedure for RNS is as detailed here: The day before the experiment, seed cells in a 96 well plate with a concentration of about 10,000 HELA cells per well. At this step, it is prudent to note that colorless media with serum should be used to allow for a higher number of healthy cells for a longer period and to avoid interference when reading the samples. Make as many cell plates as time points to ensure maximum accuracy (as the RNS is detected in the supernatant). On the day of the experiment, prepare all of the chosen concentrations of the nanoparticles and positive/negative controls. In this case, chitosan, bPEI, and bPEI-IAA (modified bPEI) are the chosen nanoparticles. The few positive controls are added for verification and baseline indication (LPF, LPS). Also leave a row open for a nitrite standard for a spectrophotometric standard curve. Once they are all added to the cell plates along with the Griess reagent and deionized water in the protocol given ratio according to the chosen plate map, place in the incubator until the marked timepoints. When the time point arrives, take the marked cell plate and add the photometric sample as well the nitrite standards. Place the cell plate back in the incubator for 30 minutes then take it out and read the absorbance of the samples. Once all of the data is received, compare the nitrite amounts to the standard curve. Repeat for each well plate and timepoint as needed. Note that the optimum wavelength for measuring absorbance is 548 nm but a range of 520-590 is also acceptable. Also note that the Griess reagent in the Invitrogen kit is made in a 1:1 ratio with components A and B (N-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid). If feasible, make an extra well plate to measure MTS at a chosen timepoint. In this experiment, the effects of serum are also tested with an extra well plate in order to see whether serum or no serum makes a difference in RNS.

Results

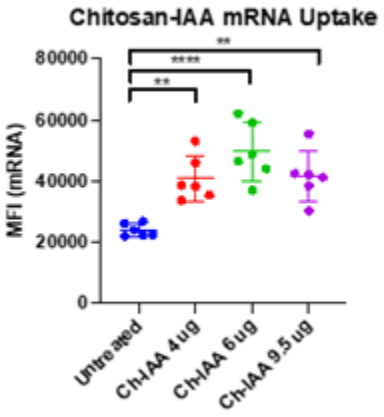

Figure 1: Observed mRNA uptake with different doses of Chitosan-IAA treated HELA cells. Each dot represents a well in a 96 well plate.

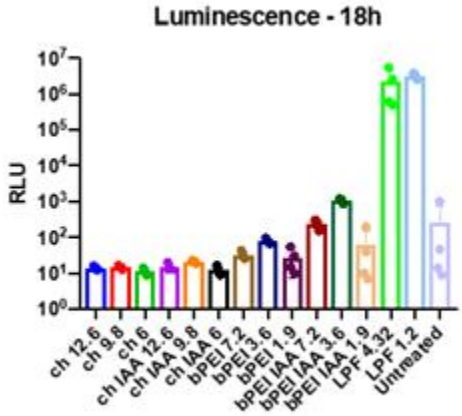

Figure 2: Luciferase mRNA expression in relative luminescence units for chitosan, chitosan-IAA, bPEI, bPEI-IAA, LPF, and untreated groups. Each dot represents a well in a 96 well plate.

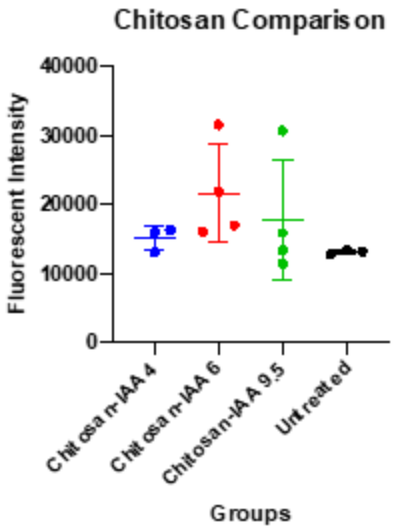

Figure 3: ROS production of different concentrations of Chitosan-IAA treated HELA cells as indicated by the DFCDA assay. Each dot represents a well in a 96 well plate.

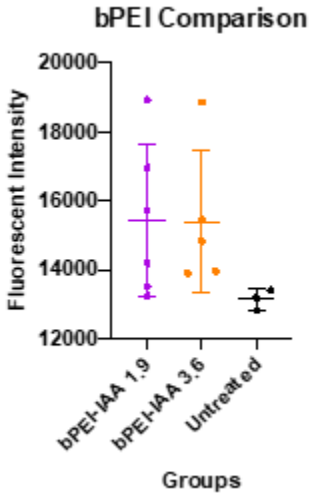

Figure 4: ROS production of different concentrations of bPEI-IAA treated HELA cells as indicated by the DFCDA assay. Each dot represents a well in a 96 well plate.

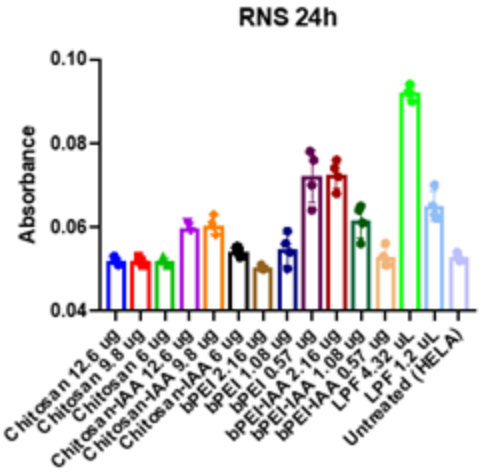

Figure 5: Absorbance RNS data after a 24-hour period for chitosan, chitosan-IAA, bPEI, bPEI-IAA, LPF, and untreated groups. Each dot represents a well in a 96 well plate.

Discussion

In Figure 1, we observed the mRNA uptake of chitosan-IAA at different doses. From the significance, it is clear that there is consistent uptake of mRNA across all the treatment doses. In Figure 2, the LPF positive control has the highest amount of mRNA expression by several orders of magnitude when compared to the rest of the treatment groups. While the mRNA expression in the chitosan groups seemed relatively consistent regardless of dose, the bPEI treatments appear to vary in their expression of mRNA depending on dose. In general, the bPEI groups have higher mRNA expression than the chitosan groups. However, none of the treatment groups came close to the relative luminescent unit values of the positive control.

The ROS experiments did not yield significant differences between chitosan-IAA and bPEI-IAA (Figures 3 and 4). While ROS is indicated to be slightly higher in the chitosan-IAA groups, it is not significant enough. But, when comparing just chitosan-IAA doses, there appears to be a notable difference between small doses as shown in Figure 3. The bPEI-IAA groups were

relatively consistent regardless of dose. The reason for this is not quite certain but it could be due to cell death or variations in ROS due to concentration.

In Figure 5, we attempted to improve upon the RNS data from a previous experiment by acquiring cleaner data. We again used the Griess assay kit to look at how RNS could be correlated to cellular transfection. We improved on the protocol by optimizing doses and coming up with a better comparison of each polymer (one high, medium, low dose). We also chose the best time point that we had observed from previous experiments and used LPF as our positive control. The data there shows a weak correlation between transfection for specific polymers but does not predict differences in transfection efficiency between them. More specifically, chitosan and chitosan-IAA groups both showed consistent RNS values within their groups. The bPEI and bPEI-IAA groups showed an interesting trend where higher doses of bPEI resulted in lower RNS values while the opposite happened for the bPEI-IAA groups. However, none of the treatment groups exhibited as much absorbance as the positive control.

Because of the previous experiments that indicated that there was uptake but little to no mRNA expression, we used the aforementioned stress granule protocols and checked uptake with flow cytometry. The stress granule experiments did not yield any feasible data due to several factors that we troubleshooted throughout the semester. The issue was that each time the cells were taken to be imaged and observed, there were either so few cells that it was impractical or that there were no cells in the wells at all. We theorized that it could be due to the numerous washing steps washing the cells out of the wells unintentionally. We also reasoned that it could be the adding of the antibodies causing issues. To solve this, we went through the protocol, as detailed above, step-by-step and checked the cells under the microscope after each one. After the first few wash steps, the cells diminished significantly. Halfway through, we found that room

temperature PBS with calcium and sodium ions must be used when there are so many washing steps in order to prevent the cells from detaching. We carried the experiment to completion using the "new" kind of PBS and found the cells to be sticking better to the plate and not washing off the way they had been.

Conclusion

Overall, polymer nanoparticles, specifically chitosan nanoparticles, exhibit potential for use in vaccines. With these results, it is clear that while uptake is seen, there is minimal expression. Cellular stress is still a possible reason why transfection does not work with chitosan nanoparticles with mRNA. Although none of the experiments we performed on reactive oxygen species, reactive nitrogen species, or stress granules showed the results we were hoping for, it is useful to know that those particular steps are not the reason. In addition, it is also possible to look into and try other modifications to chitosan that would allow for better transfection while still maintaining the same level of uptake. The results did also tell us which doses of the nanoparticles are the most optimal, and we will build upon that in the future once we are approved to do studies to use the aforementioned doses and nanoparticles for an in vivo study. We will be continuing experiments on other paths and genes of interest.

To elaborate**,** in future experiments we will be looking at other possible factors along the transfection pathway. If a particle chitosan nanoparticle can be optimized, we would also like to run in vivo studies to observe potential side effects and efficacy. For now, we will be focusing our efforts on what is shutting down transfection since that is our primary concern rather than looking at possible nanoparticles to deliver our mRNA. We will also be looking into knockout experiments which involve removing specific genes to observe their specific effects on cell

stress. If any of the target genes prove promising, we will explore possible methods of incorporating temporary gene knockout or ways to bypass triggering the specific gene to allow for more efficient transfection.

Works Cited

1. Buchan, J. R., & Parker, R. (2009). Eukaryotic stress granules: the ins and outs of translation. *Molecular cell*, *36*(6), 932-41.
2. Centers for Disease Control and Prevention. (2021). *COVID-19* U.S. Department of Health and Human Services. https://www.cdc.gov/coronavirus/2019-ncov/vaccines/faq.html#:~:text=We%20don't%20know,new%20evidence%20becomes%20available
3. del Río, L. A. (2015). ROS and RNS in plant physiology: an overview. Journal of Experimental Botany, 66(10), 2827-2837. doi:10.1093/jxb/erv099
4. Delany, I., Rappuoli, R., & De Gregorio, E. (2014). Vaccines for the 21st century. *EMBO molecular medicine*, *6*(6), 708-20.
5. Du, L., Zhou, Y., & Jiang, S. (2010). Research and development of universal influenza vaccines. Microbes and Infection, 12(4), 280-286. doi:https://doi.org/10.1016/j.micinf.2010.01.001
6. Fruehauf, J. P., & Meyskens, F. L., Jr. (2007). Reactive oxygen species: a breath of life or death? Clin Cancer Res, 13(3), 789-794. doi:10.1158/1078-0432.Ccr-06-2082
7. Garmory, H. S., Brown, K. A., & Titball, R. W. (2003). DNA vaccines: improving expression of antigens. *Genetic Vaccines and Therapy*, *1*(1), 2. doi:10.1186/1479-0556-1-2
8. Ghosn, B. (2009) Development and evaluation of an imidazole-modified chitosan for nucleic acid and contrast agent delivery. Retrieved from Texas ScholarWorks.

9. Guo, Sihan, Fu, Dongwei, Utupova, Assem, Sun, Dejun, Zhou, Mo, Jin, Zheng and Zhao, Kai. "Applications of polymer-based nanoparticles in vaccine field" *Nanotechnology Reviews*, vol. 8, no. 1, 2019, pp. 143-155. https://doi.org/10.1515/ntrev-2019-0014
10. Kim, T. K., & Eberwine, J. H. (2010). Mammalian cell transfection: the present and the future. *Analytical and bioanalytical chemistry*, *397*(8), 3173-8.
11. Moyle, P. M., & Toth, I. (2013). Modern Subunit Vaccines: Development, Components, and Research Opportunities. ChemMedChem, 8(3), 360-376. doi:10.1002/cmdc.201200487
12. Pardi, N., Hogan, M. J., Porter, F. W., & Weissman, D. (2018). mRNA vaccines — a new era in vaccinology. *Nature Reviews Drug Discovery*, *17*(4), 261-279. doi:10.1038/nrd.2017.243
13. Polack, F. P., Thomas, S. J., Kitchin, N., Absalon, J., Gurtman, A., Lockhart, S., . . . Gruber, W. C. (2020). Safety and Efficacy of the BNT162b2 mRNA Covid-19 Vaccine. *New England Journal of Medicine*, *383*(27), 2603-2615. doi:10.1056/NEJMoa2034577
14. Protter, D. S. W., & Parker, R. (2016). Principles and Properties of Stress Granules. Trends in Cell Biology, 26(9), 668-679. doi:10.1016/j.tcb.2016.05.004
15. Rezigue M. (2020) Lipid and Polymeric Nanoparticles: Drug Delivery Applications. In: Krishnan A., Chuturgoon A. (eds) Integrative Nanomedicine for New Therapies. Engineering Materials. Springer, Cham. https://doi.org/10.1007/978-3-030-36260-7_7
16. Schoenmaker, L., Witzigmann, D., Kulkarni, J. A., Verbeke, R., Kersten, G., Jiskoot, W., & Crommelin, D. J. A. (2021). mRNA-lipid nanoparticle COVID-19 vaccines: Structure and stability. *International Journal of Pharmaceutics*, *601*, 120586. doi:https://doi.org/10.1016/j.ijpharm.2021.120586